

# Roles of Pofut1 and O-Fucose in Mammalian Notch Signaling<sup>\*[S]</sup>

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Mark Stahl<sup>1,2</sup>, Kazuhide Uemura<sup>2,3</sup>, Changhui Ge<sup>4</sup>, Shaolin Shi<sup>5</sup>, Yuko Tashima<sup>6</sup>, and Pamela Stanley<sup>7</sup>

From the Department of Cell Biology, Albert Einstein College of Medicine, New York, New York 10461

Mammalian Notch receptors contain 29–36 epidermal growth factor (EGF)-like repeats that may be modified by protein O-fucosyltransferase 1 (Pofut1), an essential component of the canonical Notch signaling pathway. The *Drosophila* orthologue Ofut1 is proposed to function as both a chaperone required for stable cell surface expression of Notch and a protein O-fucosyltransferase. Here we investigate these dual roles of Pofut1 in relation to endogenous Notch receptors of Chinese hamster ovary and murine embryonic stem (ES) cells. We show that fucosylation-deficient Lec13 Chinese hamster ovary cells have wild type levels of Pofut1 and cell surface Notch receptors. Nevertheless, they have reduced binding of Notch ligands and low levels of Delta1- and Jagged1-induced Notch signaling. Exogenous fucose but not galactose rescues both ligand binding and Notch signaling. Murine ES cells lacking Pofut1 also have wild type levels of cell surface Notch receptors. However, *Pofut1*<sup>−/−</sup> ES cells do not bind Notch ligands or exhibit Notch signaling. Although overexpression of fucosyltransferase-defective Pofut1 R245A in *Pofut1*<sup>−/−</sup> cells partially rescues ligand binding and Notch signaling, this effect is not specific. The same rescue is achieved by an unrelated, inactive, endoplasmic reticulum glucosidase. Therefore, mammalian Notch receptors require Pofut1 for the generation of optimally functional Notch receptors, but, in contrast to *Drosophila*, Pofut1 is not required for stable cell surface expression of Notch. Importantly, we also show that, under certain circumstances, mammalian Notch receptors are capable of signaling in the absence of Pofut1 and O-fucose.

Notch signaling controls growth and determines cell fate in the metazoa through direct cell-cell contact (1, 2). The four mammalian Notch receptors are single pass transmembrane glycoproteins, whose extracellular domains (NECDs)<sup>8</sup> contain 29–36 tandemly organized N-terminal epidermal growth factor (EGF)-like repeats. Interaction of Notch receptors with canonical Delta or Serrate/Jagged Notch ligands expressed on neighboring cells triggers regulated intramembrane proteolytic processing that releases Notch intracellular domain (3). Upon translocation to the nucleus, Notch intracellular domain binds to CSL (CBF1/Su(H)/Lag-1), a transcriptional repressor that recruits the co-activator Mastermind, and the complex activates the expression of Notch target genes (4–6).

Numerous modulators of the canonical Notch signaling pathway have been identified, most of which act intracellularly. The discovery that Fringe, a well established modifier of Notch signaling (7), is a glycosyltransferase (8, 9) revealed that O-fucose glycans on the extracellular domain of Notch also regulate Notch signaling. EGF domains with a C<sup>2</sup>X<sub>4–5</sub>(S/T)C<sup>3</sup> consensus are substrates for protein O-fucosyltransferase 1 (Pofut1) (10). Pofut1 transfers O-fucose to Notch EGF repeats (11) and thereby generates the substrate of Fringe. The removal of Pofut1 leads to global Notch signaling defects during embryonic development in *Drosophila* (12, 13) and the mouse (14). Reduced GDP-fucose in Lec13 Chinese hamster ovary (CHO) cells (8, 15) or the wing disc (16–18) also results in Notch signaling defects. However, in mice, the inability to synthesize GDP-fucose is not manifested until after birth due to maternal rescue effects and results in failure to thrive (19). The absence of the Golgi GDP-fucose transporter Slc35c1 in mice results in a leukocyte adhesion deficiency (20), and in *Drosophila*, mutation of the orthologous gene leads to mild, temperature-sensitive Notch signaling defects (21). There may be another GDP-fucose transporter Slc35c2 (22), since the complete absence of GDP-fucose transport should lead to severe Notch signaling defects and embryonic lethality.

The mechanism by which O-fucose glycans modulate the level of Notch signaling appears, at least in part, to be by regulating ligand binding in mammals (23–26) and *Drosophila* (9, 13, 27–29). Either direct recognition of different O-fucose glycans on EGF repeats by Notch ligands and/or glycan-induced

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<sup>2</sup> Both authors contributed equally to this work.

<sup>3</sup> Supported by the Naito Foundation, Japan. Present address: Dept. of Molecular Medicine, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka 422-8526, Japan.

<sup>4</sup> Present address: Beijing Institute of Radiation Medicine, 27 Taiping Rd., Beijing 100850, China.

<sup>5</sup> Current Address: Dept. Medicine, Division of Nephrology, Mt. Sinai School of Medicine, New York, NY 10029, USA.

<sup>6</sup> Supported by The Osaka Medical Research Foundation for Incurable Diseases, Japan, and The Uehara Memorial Foundation, Japan.

<sup>7</sup> To whom correspondence should be addressed: Dept. of Cell Biology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Tel.: 718-430-3346; Fax: 718-430-8574; E-mail: [stanley@aecom.yu.edu](mailto:stanley@aecom.yu.edu).

<sup>8</sup> The abbreviations used are: NECD, Notch extracellular domain; PE, phycoerythrin; EGF, epidermal growth factor; BAPTA, 1,2-bis(o-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; DAPT, *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-5-phenylglycine *t*-butyl ester; CHO, Chinese hamster ovary; ES, embryonic stem; MFI, mean fluorescence intensity; siRNA, small interfering RNA; MES, 4-morpholineethanesulfonic acid; RNAi, RNA interference; Ab, antibody.

changes in binding strength between ligands and NECD (30, 31) may affect the initiation of downstream events leading to Notch receptor proteolysis. Although it is clear that O-fucose on Notch is an essential substrate of Fringe (8, 9), Fringe-independent Notch signaling defects are observed in *Drosophila* Ofut1 mutants (12, 13), indicative of signaling functions that are regulated solely by O-fucose on Notch. This is consistent with the reduced Jagged1-induced Notch signaling observed in Lec13 CHO cells that have very low GDP-fucose (32–35) and little Fringe activity (8, 15) and with rescue of Notch signaling by exogenous fucose or genetic complementation of the Lec13 fucosylation defect (8, 15).

Knockdown of Ofut1 in *Drosophila* S2 cells causes soluble Notch extracellular domain to be secreted poorly and to have little Notch ligand binding activity (16, 28). In addition, full-length Notch-expressing cells targeted for Ofut1 knockdown do not form aggregates with Delta-expressing S2 cells (36). However, there are conflicting reports as to whether Notch is expressed at the surface of S2 cells targeted for Ofut1 (13, 16). *In vivo*, Notch may be transiently observed at the surface of *Drosophila* epithelial cells lacking Ofut1 (37). However, there is a marked intracellular accumulation of Notch in *Ofut1*<sup>−/−</sup> mutant clones (16, 17) that has been localized to the endoplasmic reticulum by Okajima *et al.* (16, 18) and to novel endocytic vesicles by Sasaki *et al.* (37). However, there are technical concerns with the latter conclusion (38). *Drosophila* Ofut1 binds to Notch when both are overexpressed in S2 cells (16, 17), and Ofut1 facilitates soluble NECD secretion and ligand binding to S2 cells (16, 29). The secretion and folding chaperone functions of Ofut1 are retained by a fucosyltransferase-defective mutant Pofut1/Ofut1 R245A (16, 18). While this manuscript was in revision, *Drosophila* Notch synthesized in *Ofut1*<sup>−/−</sup> clones expressing *Ofut1* R245A was shown to transduce a Notch signal (18).

In this paper, we investigate the relative roles of Pofut1 and O-fucose in mediating the cell surface expression of mammalian Notch receptors as well as their abilities to bind Notch ligands and to transduce a Notch signal. We show that in murine embryonic stem (ES) cells or CHO cells, Pofut1 is not required for stable cell surface expression of mammalian Notch receptors. We also show that, under certain conditions, mammalian Notch receptors can bind Notch ligands and transduce a Notch signal in the absence of Pofut1 and O-fucose. However, active Pofut1 and O-fucosylation of Notch are required for optimal ligand binding and canonical Notch signaling induced by Delta1 or Jagged1.

## EXPERIMENTAL PROCEDURES

**Cells and Cell Culture**—CHO cells were cultured in  $\alpha$ -modified minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum (Gemini, West Sacramento, CA) unless otherwise indicated. *Pofut1*<sup>+/+</sup> or *Pofut1*<sup>−/−</sup> ES cell lines derived from blastocyst outgrowths obtained from mating *Pofut1*<sup>+/−</sup> heterozygotes as described (39) and *Notch1* null ES cells (line 290-2) kindly provided by Dr. Gregory Longmore (40) were cultured on feeder-free gelatinized plates with ES cell culture medium ( $\alpha$ -modified minimal essential medium, 10% ES-qualified fetal bovine serum (Gemini), 1000 units/ml leukemia

inhibitory factor (Chemicon, Temecula, CA), penicillin and streptomycin (Invitrogen), 50 mM  $\beta$ -mercaptoethanol (Sigma)). *Pofut1*<sup>−/−</sup> ES cells do not transfer O-fucose to EGF repeats (41). Pro<sup>−</sup>5Lec1.3C CHO cells (42) with the vector pMIRB are equivalent to parent CHO in Notch signaling activity (8, 15) and are referred to as Lec1. Lec13.6A CHO cells were previously characterized as fucosylation-defective (32–35) and are referred to as Lec13. For maximum expression of the Lec13 phenotype, Lec13 cells were cultured in  $\alpha$ -modified minimal essential medium and 10% dialyzed fetal bovine serum (Gemini). Lec1 cells expressing Lunatic fringe (Lfng) were previously described (15). Ligand cells for co-culture were L cells expressing rat Jagged1 (43) and sorted for high Jagged1 expression using goat anti-rat Jagged1 antibody AF599 (R & D Systems, Minneapolis, MN); L cells expressing rat Delta1 (23) were sorted for high Delta1 expression using goat anti-human Delta1 antibody AF 1818 (R & D Systems); and control L cells were sorted for low Jagged1 expression and shown to lack Delta1 expression.

**Preparation of Delta1 and Jagged1 Notch Ligands**—An expression construct encoding rat Delta1-Fc kindly provided by Dr. Gerry Weinmaster (23), was stably expressed in HEK293T cells, and a population producing  $\sim 10$ – $16$   $\mu$ g/ml Delta1-Fc was isolated. The expression construct for rat Jagged1-Fc also from Dr. Gerry Weinmaster (23) was inserted into pIRES2-EGFP (Clontech). After stable expression in HEK293T cells, a population producing  $\sim 6$   $\mu$ g/ml was isolated by sequential enrichment using flow cytometry sorting. For ligand preparation, HEK293T ligand-expressing cells were cultured in suspension using Pro293a serum-free medium (Cambrex Bio Science, Rockland, ME). Culture supernatants were assayed by Western blot using horseradish peroxidase-conjugated anti-human IgG (Zymed Laboratories Inc., South San Francisco, CA). Each ligand gave a single band with apparent molecular masses of  $\sim 82$  kDa (Delta1-Fc) and  $\sim 180$  kDa (Jagged1-Fc), respectively. Concentrations of Delta1-Fc and Jagged1-Fc in medium were estimated by Western blot densitometry compared with an IgG standard using NIH Image software. After sterile filtering, ligands were stable for several months at 4 °C.

**Notch1 ECD Fragment Preparation**—Notch1 ECD fragment was produced in Lec1 cells stably transfected with mammalian expression construct pSectag mNotchEGF-(1–18)(wt)MycHis<sub>6</sub> kindly provided by Dr. Robert Haltiwanger (44), cultured for  $\sim 96$  h in Opti-MEM I reduced serum medium (Invitrogen) supplemented with 1 mM CaCl<sub>2</sub>, penicillin, and streptomycin. The medium was clarified by centrifugation, and the supernatant was rotated with Ni<sup>2+</sup>-nitrilotriacetic acid-agarose (Qia-gen, Hilden, Germany) at pH 8.0 overnight at 4 °C (750 ml of medium, 5 ml of resin). After washing, the fragment was eluted with 500 mM imidazole. Silver staining and Western blot analysis identified fractions containing the highest amount of Notch1EGF-(1–18) which was dialyzed against fragment buffer (20 mM Tris, 150 mM NaCl, 1 mM CaCl<sub>2</sub>, pH 7.4), concentrated using a Centricon YM-50 filter (50 kDa; Millipore, Billerica, MA), and stored at  $-80$  °C.

## Notch Receptors Lacking O-Fucose May Signal

**Notch Ligand and Antibody Binding Assays by Flow Cytometry**—CHO cells growing in suspension were harvested by centrifugation, washed, and resuspended in ligand binding buffer (Hanks' balanced salt solution (Mediatech Inc., Herndon, VA) to which 1 mM  $\text{CaCl}_2$ , 1% bovine serum albumin, and 0.05%  $\text{NaN}_3$ , pH 7.4, were added). In experiments involving transient transfection prior to the binding assay, CHO cells were grown in monolayer, and ES cells were grown under feeder-free conditions on gelatinized 100-mm plates. ES cells were transfected with 3.5  $\mu\text{g}$  of mouse Pofut1 cDNA (39), a mouse Pofut1 cDNA generated by site-directed mutagenesis to contain the point mutation R245A (16), an  $\alpha$ -glucosidase I cDNA containing the S440F point mutation (45), or vector alone (pcDNA3.1; Invitrogen) using Lipofectamine 2000 (Invitrogen). Lec13 CHO cells were grown in 6-well plates and transfected using FuGENE 6 (Roche Applied Science) with 1  $\mu\text{g}$  of a mouse Notch1 cDNA or empty vector (pCS2) kindly provided by Dr. Raphael Kopan. At 30–36 h post-transfection, cells were dissociated from plates using phosphate-buffered saline-based enzyme-free cell dissociation solution (Chemicon, Temecula, CA) to preserve the integrity of cell surface proteins. Cells ( $5 \times 10^5$ ) were incubated with soluble Notch ligands (Delta1-Fc or Jagged1-Fc) or anti-NECD antibodies in 0.2 ml of ligand binding buffer for 30–60 min at room temperature or at 4 °C with gentle rotation. Sodium azide blocked endocytosis that may occur at room temperature. Alternatively, cells were washed and then fixed with 4% paraformaldehyde in phosphate-buffered saline for 15 min at room temperature before adding primary antibody. After washing with binding buffer three times, cells were incubated with secondary antibody in binding buffer for 30 min at the binding temperature. Cells were washed with 1 ml of binding buffer three times, 1  $\mu\text{g}/\text{ml}$  propidium iodide or 7-amino-actinomycin D (BD Pharmingen, San Diego, CA) was added, and the cells were subjected to flow cytometric analysis using a FACScan or FACSCalibur (BD Biosciences, San Jose, CA) instrument. Fluorescence-activated cell sorting data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR) and are presented as profiles obtained after exclusion of dead or damaged cells that took up propidium iodide or 7-amino-actinomycin D.

For Notch1 fragment inhibition studies, ligand binding was carried out as above, except that soluble Notch ligand was pre-incubated for 30 min at room temperature with Notch1 EGF-(1–18) fragment (100  $\mu\text{g}$ ) before mixing with cells. An equal amount of fragment buffer (20 mM Tris, 150 mM NaCl, 1 mM  $\text{CaCl}_2$ , pH 7.4) was added to controls that did not contain the Notch1 fragment.

Notch ECD antibodies and dilutions were as follows: hamster anti-Notch1 (1:10; clone 8G10; Upstate Biotechnology, Inc., Lake Placid, NY); rabbit anti-Notch2 (1:100; sc-5545 against amino acids 25–255 human NOTCH2; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); goat anti-Notch3 ECD (1:50–100; AF1308; R&D Systems, Minneapolis, MN); monoclonal antibody 5E1 against human NOTCH3 ECD (1:20 culture supernatant (46)); goat anti-Notch4 ECD (1:100; clone L-16; sc-8645; Santa Cruz Biotechnology). Secondary antibodies were R-phycoerythrin (PE)-conjugated goat anti-human IgG F(ab')<sub>2</sub> fragment (Jackson ImmunoResearch, West Grove, PA), anti-ham-

ster-Alexa488 (Molecular Probes, Inc., Carlsbad, CA), anti-rabbit IgG-PE, anti-goat IgG-PE, or anti-mouse IgG-PE (all from Jackson ImmunoResearch and used at 1:100 or a lower dilution).

**Fixed and Intracellular Notch Receptors**—ES cells growing on gelatinized plates in ES cell medium were collected by enzyme-free cell dissociation solution (Chemicon) and washed in ligand binding buffer. To examine binding of anti-Notch3 antibodies to fixed and permeabilized ES cells, washed cells ( $1 \times 10^6$ ) were fixed with Fix & Perm Reagent A (Fixation Medium; Caltag Laboratories, Burlingame, CA) for 15 min at room temperature, centrifuged, resuspended in 1 ml of binding buffer, and divided into two aliquots. After centrifugation, half the cells were incubated in 100  $\mu\text{l}$  of Reagent B (Permeabilization Medium), and the other half were incubated in 100  $\mu\text{l}$  of binding buffer for 30 min at room temperature. After centrifugation, cells were resuspended in 100  $\mu\text{l}$  of binding buffer or 100  $\mu\text{l}$  of binding buffer containing primary anti-Notch3 antibody (1:20 clone 5E1 (46) or 1:10 clone AF1308 (Santa Cruz Biotechnology)) and incubated for 60 min at room temperature. After centrifugation and washing twice with 0.45 ml of binding buffer, cells were incubated in PE-labeled goat anti-mouse or anti-rabbit IgG (1:100) in binding buffer for 20 min at room temperature. Cells were centrifuged, washed, and resuspended in binding buffer for flow cytometric analysis.

To examine the effects of tunicamycin on intracellular Notch accumulation, ES cells growing in feeder-free culture on gelatinized plates were treated with 2  $\mu\text{g}/\text{ml}$  tunicamycin (Sigma) dissolved in DMSO or DMSO vehicle for 6 h before being collected in cell dissociation solution and washed in ligand binding buffer containing sodium azide. Then  $1 \times 10^6$  cells were incubated with either anti-Notch3 antibody (1:50; AF1308; R&D Systems) for 30 min at room temperature to label cell surface Notch3 or in PE-labeled secondary antibody as control. After washing, cells were incubated with PE-labeled secondary antibody in binding buffer for 15 min at room temperature and then in Fix & Perm Reagent A (Fixation Medium) according to the manufacturer's instructions (Caltag Laboratories, Burlingame, CA). Cells were washed and divided into two aliquots. To one was added Fix & Perm Reagent B (Permeabilization Medium) containing anti-Notch3 antibody (1:50) in binding buffer, and to the other was added binding buffer alone with anti-Notch3 antibody AF1308 (1:50). Cells were incubated for 30 min at room temperature, washed, and incubated with PE-labeled secondary antibody in either Fix & Perm Reagent B (Permeabilization Medium) in binding buffer or binding buffer alone, respectively, and incubated for 15 min at room temperature. After centrifugation, cells were resuspended in binding buffer for flow cytometric analysis. Mean fluorescence intensity (MFI) was used to calculate the percentage binding of anti-Notch3 ECD antibodies to permeabilized *versus* nonpermeabilized cells.

**Ligand-dependent Notch Signaling Assays**—Notch signaling assays were performed as previously described (15, 39). Briefly, for CHO cells, duplicate cultures were plated at  $2 \times 10^5$  cells/well in 6-well plates and cotransfected the next day using FuGENE 6 (Roche Applied Science) with 0.2  $\mu\text{g}$  of the plasmid TP-1 that carries eight copies of a RBP-J $\kappa$  DNA binding

sequence driving a firefly luciferase reporter gene (47), 0.05  $\mu\text{g}$  of pRL-TK *Renilla* luciferase (Promega, Madison, WI), and 1.5  $\mu\text{g}$  of pMIRB empty vector. After 16 h at 37 °C, 1.5–2  $\times 10^6$  Jagged1/L, Delta1/L, or control L cells were overlaid. After another 30 h, firefly and *Renilla* luciferase activities were quantitated in cell lysates using a dual luciferase assay (Promega, Madison, WI). Ligand-dependent Notch activation is expressed as -fold induction of normalized luciferase activity stimulated by ligand/L cells compared with L cells.

For ES cell experiments, duplicate cultures were plated at  $2.5 \times 10^5$  cells/well of a 6-well plate in ES cell culture medium and, after ~16 h, were cotransfected with 0.2  $\mu\text{g}$  of TP1, 0.05  $\mu\text{g}$  of pRL-TK *Renilla* luciferase reporter plasmid, and 3.8  $\mu\text{g}$  of the indicated cDNA construct or empty vector using Lipofectamine 2000 (Invitrogen). At 16 h post-transfection,  $10^6$  ligand-expressing (Jagged1/L or Delta1/L) or control L cells were overlaid. At 48 h after transfection, luciferase and *Renilla* luciferase activities in lysates were measured by the dual luciferase assay, and -fold induction was calculated as above.

**Ligand-independent Notch Signaling Assay**—ES or CHO cells at ~50% confluence in 6-well plates were cotransfected in duplicate with 0.2  $\mu\text{g}$ /well TP-1 plasmid and 0.05  $\mu\text{g}$ /well pRL-TK using Lipofectamine 2000 (Invitrogen). At 16–24 h after transfection, cells were briefly treated with Hanks' balanced salt solution containing various concentrations of the calcium chelators EDTA or BAPTA (Molecular Probes) or 2.5 mM  $\text{CaCl}_2$  for 5–30 min followed by incubation in growth medium for 6–8 h. To ensure specificity for canonical Notch signaling, cells were treated with chelator and the  $\gamma$ -secretase inhibitor DAPT (Calbiochem) to a final concentration of 500 nM in DMSO or DMSO alone (vehicle) during chelator treatment and the subsequent incubation period. To demonstrate that the chelation effect was on Notch ECD, cells expressing constitutively active Notch1 lacking the ECD (ZEDN1 (11); kindly provided by Dr. Gerry Weinmaster) were compared for Notch signaling in 2.5 mM  $\text{CaCl}_2$  and after chelation. Firefly and *Renilla* luciferase activities were determined in whole cell lysates using a dual luciferase assay (Promega, Madison, WI). -Fold induction is given as the ratio of luciferase activity of EDTA- or BAPTA-treated cells compared with cells incubated in 2.5 mM  $\text{CaCl}_2$  after normalization.

**Targeted Knockdown of Pofut1 in Lec1 Cells**—siRNAs to eliminate Pofut1 transcripts in CHO cells were produced with the Silencer<sup>®</sup> siRNA construction kit (Ambion, Austin, TX). Six different siRNAs targeting the coding region of a CHO Pofut1 cDNA were tested by transient transfection followed by Northern analysis using a Pofut1 coding region probe. The sequences 5'-AAGTCCTGATAAGAAGACATG-3' and 5'-AACCTCCTTTCACTAATCTCC-3' produced the most significant reductions in Pofut1 transcript levels, and were cloned into the vectors pSilencer2.1-U6 neo and pSilencer2.1-U6 hyg, respectively. These constructs were transfected either individually or in combination into Lec1 cells using Lipofectamine 2000 (Invitrogen), and selection for stable transfectants was performed using G418 (neomycin) at 1.5 mg/ml active concentration (Gemini, West Sacramento, CA) or hygromycin at 500  $\mu\text{g}$ /ml (Calbiochem) or both together as appropriate. The cell lines developed were termed HN2 and HN9 (containing both

targeting sequences) and HY4 containing only the 5'-AACCTCCTTTCACTAATCTCC-3' sequence.

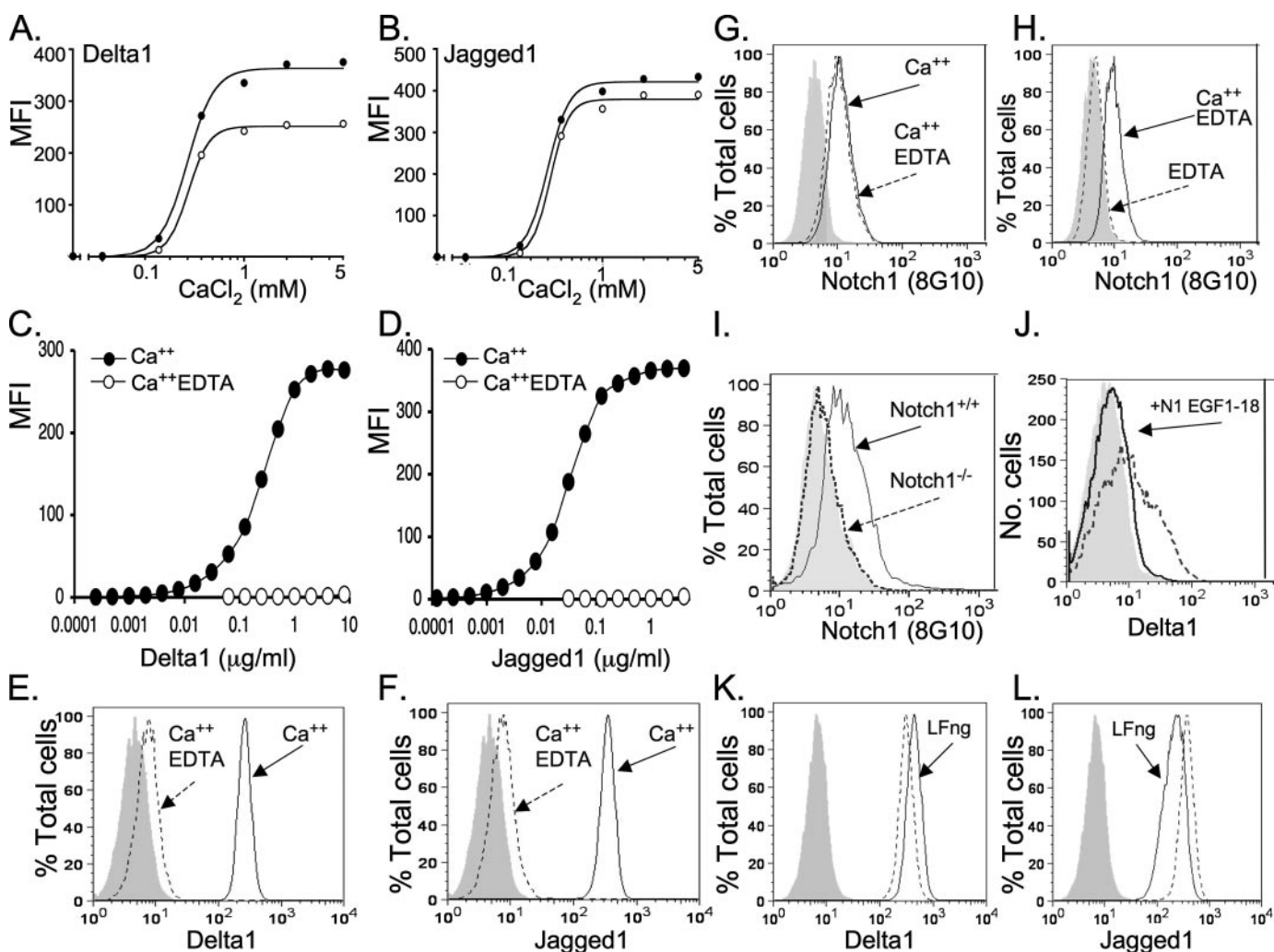
**Northern Blot Analysis**—Total RNA from CHO cells stably transfected with Pofut1 siRNA constructs was prepared using TRIZOL (Invitrogen), electrophoresed, transferred to a nylon membrane, and hybridized for 2 h in Rapid-hyb buffer (Amersham Biosciences) containing a  $^{32}\text{P}$ -labeled mouse Pofut1 cDNA coding region probe at 65 °C. The blot was washed with 2 $\times$ , 0.5 $\times$ , and 0.1 $\times$  SSC, 0.1% SDS solution, respectively, each for 20 min at 65 °C. X-ray film exposure was overnight at –80 °C.

**Whole Cell Lysate Preparation**—Cells ( $10^7$ ) were washed with phosphate-buffered saline, pH 7.4, resuspended in 75  $\mu\text{l}$  of lysis buffer (1.5% Triton X-100 and 1 $\times$  Complete<sup>™</sup> EDTA-free protease inhibitor (Roche Applied Science), vortexed, and incubated on ice for 10 min. Lysates were centrifuged at 6000  $\times g$  for 5 min, and the supernatant was collected. Total protein concentration was assayed using the Bio-Rad Dc protein assay kit (Bio-Rad) according to the manufacturer's instructions.

**Western Blot Analysis**—Protein (~50  $\mu\text{g}$ ) was separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Blocking was performed in TBST (25 mM Tris-HCl, 125 mM NaCl, 0.1% Tween 20, pH 7.4) containing 5% nonfat dry milk. Primary and secondary antibodies were diluted in TBST containing 5% nonfat dry milk and incubated with the blot for 2 h and 30 min, respectively. Proteins were visualized using enhanced chemiluminescence (SuperSignal kit; Pierce). Anti-bovine Pofut1 rabbit antibody was a kind gift from Drs. Celine Lorient and Abderrahman Maftah (48). Secondary antibody for Pofut1 was horseradish peroxidase-conjugated anti-rabbit IgG (Stressgen, Ann Arbor, MI).

**Pofut1 and  $\beta$ 4-Galactosyltransferase Assays**—Pofut1 activity was measured in cell lysates essentially as described (11). Each 50- $\mu\text{l}$  reaction contained a final concentration of 100 mM imidazole-HCl, pH 7.0, 50 mM  $\text{MnCl}_2$ , 0.1 mM GDP-[ $^{14}\text{C}$ ]fucose (4000–8000 cpm/nmol), 20  $\mu\text{M}$  recombinant human Factor VII EGF-1 domain kindly provided by Dr. Robert Haltiwanger, and 40 or 80  $\mu\text{g}$  of total protein from cell extracts and incubated at 37 °C for 30 min. Reactions lacking acceptor were used as a control. Reactions were stopped by the addition of 950  $\mu\text{l}$  of cold 0.25 M EDTA, pH 8.0. [ $^{14}\text{C}$ ]Fucose transferred to the EGF repeat was identified by passage of the reaction mix through an Accubond II C18 cartridge (Agilent Technologies, Santa Clara, CA), washing with 5 ml of  $\text{H}_2\text{O}$ , and eluting the EGF repeat with 3 ml of 80% acetonitrile containing 0.052% trifluoroacetic acid. Radioactivity was measured in a liquid scintillation counter.

$\beta$ 4-Galactosyltransferase activity was measured in cell lysates essentially as described (49). Reactions contained 100 mM MES buffer at pH 6.5, 60 mM  $\text{MnCl}_2$ , 1.2% Triton X-100, 35 nmol of UDP-[6- $^3\text{H}$ ]Gal (~10,000 cpm/nmol), 10 mM *N*-acetylglucosamine (acceptor), and ~100  $\mu\text{g}$  of total protein from cell extract in 50  $\mu\text{l}$ . Reactions lacking acceptor were used as a control. After incubation at 37 °C for 2 h, the reaction was stopped by adding 1 ml of cold water. Reactions were passed over a 1-ml column of AG 1-X4 ( $\text{Cl}^-$  form) (Bio-Rad), and unbound product was collected in 2 ml of water. Radioactivity was measured in a liquid scintillation counter.



**FIGURE 1. Notch ligand binding to CHO cells.** A, Notch ligand binding is dependent on  $\text{Ca}^{2+}$  concentration. Binding of 2  $\mu\text{g/ml}$  (filled circles) and 0.5  $\mu\text{g/ml}$  (open circles) soluble Delta1-Fc to Lec1 cells over a range of calcium concentrations was determined by flow cytometry. Data are MFI with ligand and secondary antibody minus MFI with secondary Ab alone. The optimal least squares fits of Hill's equation ( $Y = A \times X^N / (K_{0.5}^N + X^N)$ ) (solid lines) are shown. B, binding of 0.5  $\mu\text{g/ml}$  (filled circles) and 0.125  $\mu\text{g/ml}$  (open circles) soluble Jagged1-Fc to Lec1 cells as in A. C, binding of soluble Delta1-Fc to Lec1 cells over a range of Delta1-Fc concentrations by flow cytometry. Filled circles, incubation in buffer containing 1 mM  $\text{CaCl}_2$ ; open circles, incubation in buffer containing 1 mM  $\text{CaCl}_2$  and 5 mM EDTA. Data are MFI with ligand and secondary antibody minus MFI with secondary Ab alone. D, binding of Jagged1-Fc to Lec1 cells as in C. E, binding of 8  $\mu\text{g/ml}$  soluble Delta1-Fc to Lec1 cells analyzed by flow cytometry. The shaded profile is secondary antibody alone, the solid profile is for ligand in buffer containing 1 mM  $\text{CaCl}_2$ , and the dashed profile is ligand binding in buffer containing 1 mM  $\text{CaCl}_2$  and 5 mM EDTA. F, binding of 4  $\mu\text{g/ml}$  soluble Jagged1-Fc to Lec1 cells as in E. G, anti-Notch1 ECD antibody 8G10 binding to Lec1 cells incubated in binding buffer with 1 mM  $\text{CaCl}_2$  (solid line) or 1 mM  $\text{CaCl}_2$  and 5 mM EDTA (dashed line). The shaded profile is for secondary antibody alone. H, Notch1 antibody binding to Lec1 cells in buffer containing 5 mM EDTA with 1 mM  $\text{CaCl}_2$  (solid line) or EDTA without calcium (dashed line). The shaded profile is for secondary antibody alone. I, antibody 8G10 is specific for Notch1. Anti-Notch1 ECD antibody 8G10 binding to Notch1<sup>+/+</sup> ES cells (solid line) compared with Notch1<sup>-/-</sup> ES cells (dashed line) was analyzed by flow cytometry. The shaded profile is secondary antibody alone. J, a Notch1 ECD fragment inhibits Delta1-Fc binding. Notch1 fragment EGF-(1-18) (N1-18) prepared in Lec1 CHO cells was preincubated with soluble Delta1-Fc (0.5  $\mu\text{g/ml}$ ) for 30 min before the mixture was incubated with Pofut1<sup>+/+</sup> ES cells and analyzed by flow cytometry (solid line). The dashed profile is binding of Delta1-Fc in the absence of N1-18, and the shaded profile is for secondary antibody alone. K, ligand binding to Lec1 cells is modulated by Fringe. The shaded profile is for secondary antibody alone, the solid profile is for binding of 2  $\mu\text{g/ml}$  soluble Delta1-Fc to Lec1 cells stably expressing Lfng, and the dashed line is for binding of Delta1-Fc to Lec1 cells stably expressing control vector. L, soluble Jagged1-Fc (0.5  $\mu\text{g/ml}$ ) binding to Lec1 cells expressing Lfng (solid line) or control vector (dashed line).

## RESULTS

**Binding of Delta1 and Jagged1 to CHO Cell Surface Notch Receptors**—The binding of soluble Notch ligands Delta1-Fc or Jagged1-Fc to Notch receptors at the surface of CHO cells was analyzed by flow cytometry, which has the advantages of high sensitivity, high specificity, and the ability to view a large number of cells in a population. In addition, the gating out of 7-amino-actinomycin D-positive cells that have a compromised plasma membrane means that all binding detected in our experiments reflects cell surface binding. Ligand binding to the Lec1 CHO mutant that expresses a simplified complement of

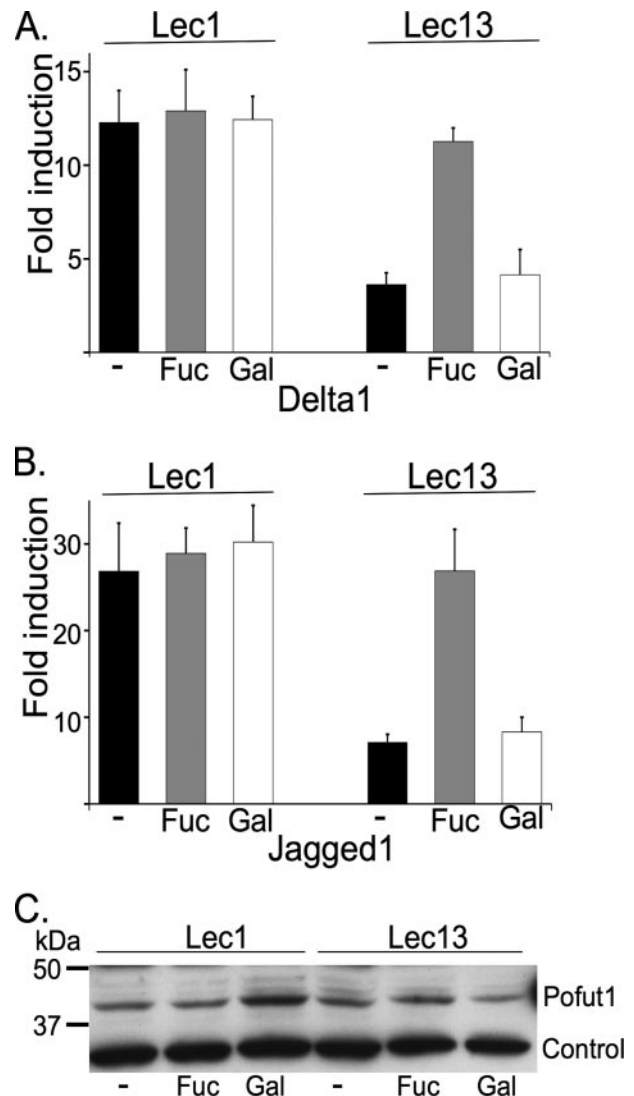
N-glycans but a wild type complement of O-fucose glycans (15, 50) is shown in Fig. 1, A–F. These experiments were performed in binding buffer containing calcium and sodium azide to prevent endocytosis at room temperature. There was no difference between 0.05 and 0.1% azide in the binding buffer. Consistent with previous reports (23–25, 30, 51), Notch ligand binding was calcium-dependent (Fig. 1, A and B). The calcium dependence curve fit well to Hill's equation ( $R^2 > 0.998$ ), and the value of Hill's coefficient for both ligands was 1.2. This indicates that Delta1-Fc and Jagged1-Fc bound to Notch receptors in a non-cooperative manner. In buffer containing 1 mM  $\text{CaCl}_2$ , soluble

Delta1 and Jagged1 bound to Lec1 CHO cells with an approximate  $K_d$  of  $9.1 \times 10^{-10}$  and  $3.3 \times 10^{-9}$  M, respectively (Fig. 1, C and D). These values are similar to those reported by Shimizu *et al.* (25). The binding of Delta1 and Jagged1 was abrogated by the addition of 5 mM EDTA to the binding buffer (Fig. 1, C–F). Antibodies to the extracellular domain of Notch1 were used to determine if this treatment released NECD (Fig. 1G). Under optimal ligand binding conditions at room temperature or 4 °C (not shown), anti-Notch1 ECD bound well to the Lec1 cell surface (Fig. 1G). Notch1 antibody binding was only slightly reduced by the addition of 5 mM EDTA to the binding buffer (Fig. 1G), the condition that markedly inhibited the binding of Notch ligands (Fig. 1, E and F). Therefore, incubation with binding buffer containing 1 mM  $\text{CaCl}_2$  and 5 mM EDTA released few Notch receptors from the cell surface but prevented ligand binding. However, in the absence of  $\text{CaCl}_2$ , incubation in 5 mM EDTA released Notch1 receptors from the cell surface (Fig. 1H), as reported previously (30, 51).

The specificity of the anti-Notch1 ECD antibody (8G10) was verified by showing that binding to *Notch1* null ES cells (40) was negligible (Fig. 1I). The specificity of monoclonal antibody 8G10 for Notch1 was also apparent from Western analysis (see Fig. 5E). Notch ligand binding specificity was verified by showing that a soluble Notch1 fragment that includes the N-terminal EGF repeats 1–18 inhibited the binding of Delta1 to ES cells (Fig. 1J). The same Notch1 fragment also inhibited Jagged1 binding to ES cells but less markedly (not shown). Jagged1 may bind better to other Notch receptors on mouse ES and CHO cells (see Fig. 5 and Fig. S3). Another measure of the specificity of the Delta1 and Jagged1 ligands for Notch receptors was obtained using Lec1 cells expressing Lunatic Fringe (Lfng). Lfng enhanced the binding of Delta1 (Fig. 1K) and inhibited the binding of Jagged1 (Fig. 1L), similar to previous observations with mammalian cells (23, 24, 30).

**Optimal Notch Ligand Binding and Notch Activation Requires O-Fucosylation of Notch**—Lec13 CHO cells have a mutation in the gene that encodes GDP-Man-4,6-dehydratase (Gmd) (33, 34). These papers reported no detectable Gmd transcripts or GDP-fucose in Lec13 cells, but a recent analysis found that Lec13 has about 3% of the GDP-fucose levels in CHO cells (35). Since Lec13 cells are rescued by exogenous fucose added to the culture medium (32), culture conditions affect the level of fucosylation. After 4 days of growth in dialyzed fetal calf serum, fucosylated cell surface glycoproteins are very low in Lec13 cells (15).

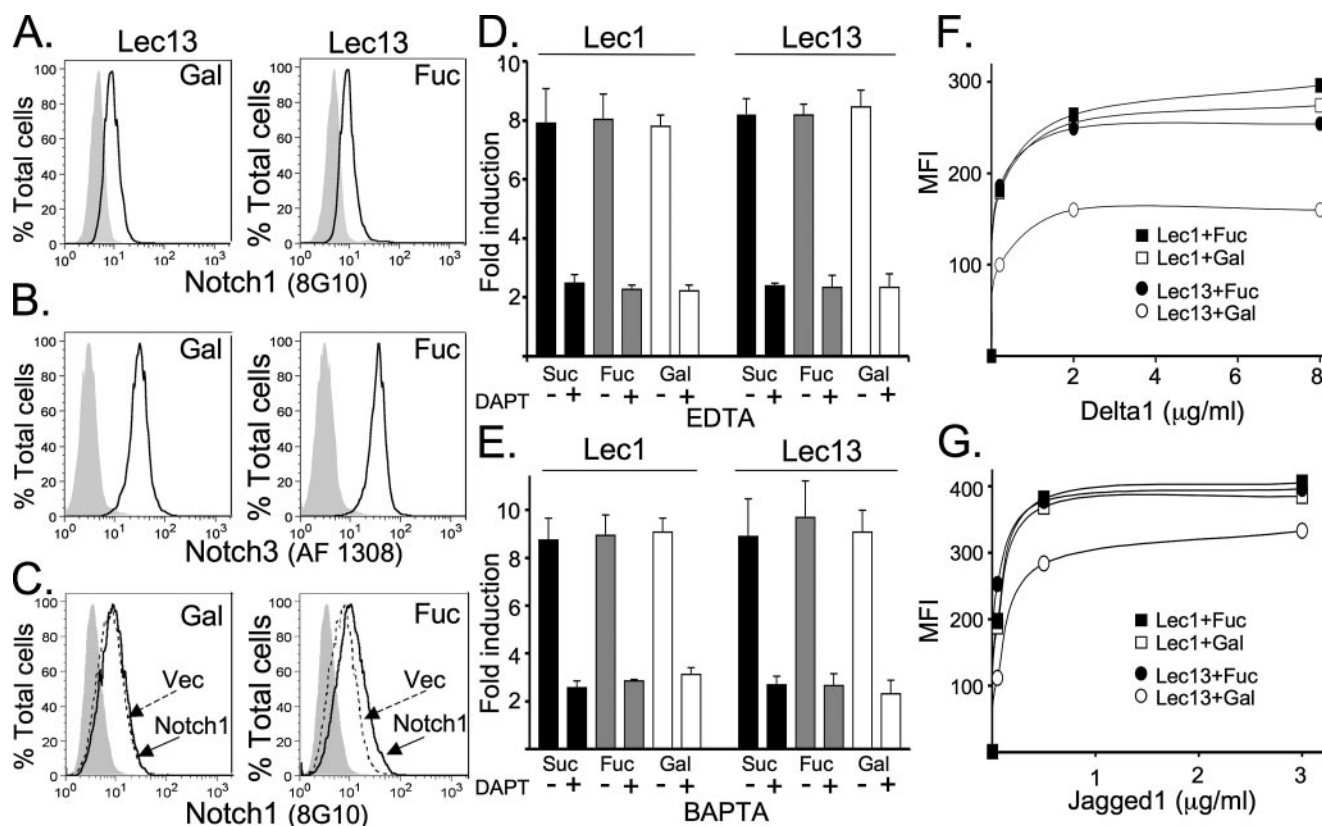
We previously showed that Jagged1-induced Notch signaling is reduced in Lec13 CHO cells using a CBF1-luciferase reporter (8, 15). This co-culture assay was significantly improved by substituting a TP-1-luciferase Notch signaling reporter (47) and by using ligand-expressing cells sorted for high expression of Delta1 (Fig. S1) or Jagged1 (15), respectively. In the absence of exogenous fucose, Delta1-induced Notch signaling was low in Lec13 cells (Fig. 2A). The addition of 1 mM fucose, but not 1 mM galactose, to culture medium rescued signaling in Lec13 but did not affect signaling in Lec1 cells (Fig. 2A). Similar results were obtained for Jagged1-induced Notch signaling (Fig. 2B). The level of Pofut1 determined by Western analysis (Fig. 2C) or



**FIGURE 2. Lec13 cells are deficient in ligand-induced Notch signaling but express Pofut1.** Lec1 and Lec13 cells were cultured in medium containing no addition (–) or 1 mM fucose (Fuc) or 1 mM galactose (Gal) for 4 days. A, Delta1-induced Notch signaling in Lec1 and Lec13 cells. –Fold induction for Delta1/L:L was calculated after normalization. Error bars, S.D. ( $n = 6$ ). B, Jagged1-induced Notch signaling in Lec1 and Lec13 cells. –Fold induction for Jagged1/L:L was calculated after normalization. Error bars, S.D. ( $n = 6$ ). C, cell lysates (50  $\mu$ g of protein) were analyzed by Western blot using bovine anti-Pofut1 antibodies (diluted 1:500). Control is a nonspecific band on the same blot.

Pofut1 activity ( $\sim 7$  nmol/h/mg protein) was equivalent in Lec13 cells under all growth conditions.

The fucose dependence of Notch signaling in Lec13 cells may be due to a requirement for O-fucose on Notch receptor EGF repeats or to increased expression of Notch receptors at the surface of Lec13 cells grown in fucose. To investigate the latter, the levels of cell surface Notch1 and Notch3 were investigated using anti-NECD antibodies and flow cytometry in Lec1 (not shown) and Lec13 cells grown in the presence and absence of exogenous fucose or galactose. Sugar supplementation had essentially no effect on cell surface levels of Notch1 (Fig. 3A) or Notch3 (Fig. 3B) in either cell line. Lec13 cells exhibited a small increase in the amount of Notch1 at the cell surface when transiently transfected with a Notch1 expression construct in

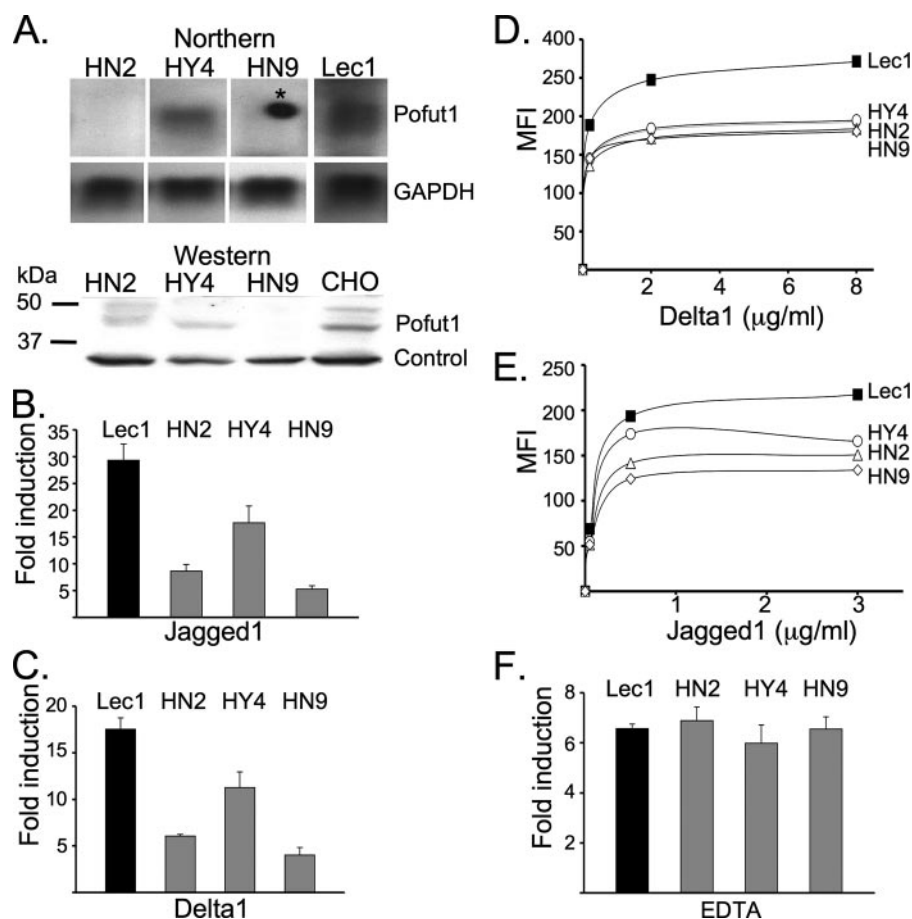


**FIGURE 3. Fucose supplementation rescues Notch ligand binding but does not affect cell surface expression of endogenous Notch receptors in Lec13 cells.** Lec13 cells grown in medium supplemented with 1 mM fucose or 1 mM galactose were incubated with Notch1 extracellular domain antibody (8G10) (A) or Notch3 extracellular domain antibody (AF1308) (B) and analyzed by flow cytometry. The shaded profiles are secondary antibody alone. C, Lec13 cells cultured in 1 mM Gal or 1 mM Fuc were transfected with Notch1 (solid profile) or empty vector (dashed profile) and analyzed for binding of anti-Notch1 ECD antibody 8G10 by flow cytometry. D, ligand-independent signaling following calcium depletion by EDTA. Cells were cultured for 4 days in medium containing 1 mM fucose, 1 mM galactose, or 1 mM sucrose and cotransfected with the TP-1 Notch-responsive luciferase and control pRL-TK *Renilla* luciferase reporters. After 16 h, cells were incubated in Hanks' balanced salt solution containing 4 mM EDTA or 2.5 mM  $\text{CaCl}_2$  with or without the  $\gamma$ -secretase inhibitor DAPT (500 nM) for 5 min and then in complete medium for 6 h before assessing Notch activation by a dual luciferase assay. -Fold induction is the ratio of normalized EDTA-treated to  $\text{CaCl}_2$ -treated cells. Error bars, S.D. ( $n = 4$ ). E, ligand-independent signaling following calcium depletion by 7.5 mM BAPTA performed as described in D. F, Lec13 cells are deficient in Notch ligand binding and rescued by fucose. Lec1 cells (squares) and Lec13 cells (circles) were cultured for 4 days in 1 mM fucose (solid) or 1 mM galactose (open) and tested for soluble Delta1-Fc binding by flow cytometry. Each data point indicates MFI of primary and secondary antibody minus binding to secondary Ab alone. G, soluble Jagged1 binding to Lec1 and Lec13 cells performed as described in F.

medium with galactose (Fig. 3C). However, if the transient transfection was performed in medium supplemented with 1 mM fucose, a somewhat greater increase in cell surface Notch1 was observed (Fig. 3C). This suggests that O-fucosylation of Notch1 may slightly facilitate trafficking of Notch receptors to the CHO cell surface when Notch1 is overexpressed. Similarly, overexpressed Notch1 ECD fragments are secreted more efficiently from Lec13 cells if fucose is present (data not shown). Clearly, however, the reduced signaling of endogenous Notch receptors in Lec13 cells (Fig. 2, C and D) was not due to reduced expression of endogenous Notch1 or Notch3 receptors at the cell surface. Similar results were obtained with anti-Notch2 and anti-Notch4 ECD antibodies (not shown). The low level of Notch signaling in Lec13 cells grown in the absence of fucose may reflect the presence of a small amount of O-fucose on Notch receptors.

As an independent measure of Notch receptors at the surface of Lec13 cells, a ligand-independent assay of Notch signaling was performed (51). All four mammalian Notch receptors are maintained at the cell surface as heterodimers. This association is calcium-dependent, and removal of calcium from the medium causes cultured cells to shed NECD (51). NECD shed-

ding, in turn, allows proteases to act on the Notch transmembrane domain, leading to release of the Notch intracellular domain, which translocates to the nucleus and activates Notch target genes. This constitutes a ligand-independent Notch signaling assay that reflects the number of Notch receptors at the cell surface. For this assay, the Notch TP-1-luciferase reporter and pRL-TK-*Renilla* control were transfected into Lec1 and Lec13 cells previously grown in the presence of 1 mM sucrose, 1 mM galactose, or 1 mM fucose. After 24 h, the cells were treated briefly with EDTA or BAPTA to chelate calcium or maintained in 2.5 mM  $\text{CaCl}_2$ , followed by culture for 6–8 h before determination of luciferase activities. Conditions that caused maximal ligand-independent Notch signaling were determined (Fig. S2), and concentrations of chelator and times of incubation were chosen so that signaling was approximately half-maximal. The assay was shown to be specific for Notch at the cell surface, because ZEDN1, which lacks the Notch1 ECD (11), was found to be similarly active in the presence and absence of chelators, as would be predicted (Fig. S2). Inhibition of Notch signaling by the  $\gamma$ -secretase inhibitor DAPT showed that the assay was specific for Notch receptor activation (Fig. S2). Antibodies to the NECD of Notch1, Notch2, Notch3, or Notch4 bound poorly to



**FIGURE 4. CHO cells deficient in Pofut1 have reduced Notch signaling and ligand binding.** A, total RNA from Lec1 or Lec1 cell lines stably expressing RNAi targeting Pofut1 transcripts was analyzed by a Northern blot hybridized to a probe from the *Pofut1* gene coding region (top). The same blot was stripped and hybridized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The star indicates a nonspecific signal. Detergent lysates prepared from the same cell lines were analyzed by Western blot for Pofut1 protein (bottom). Control is a nonspecific band on the same gel. B, Lec1 cells expressing RNAi targeted against Pofut1 transcripts were assayed for Notch signaling induced by co-culture with Jagged1/L compared with control L cells. Bars, -fold induction of signaling with S.D. ( $n = 4$ ). C, Lec1 cells expressing RNAi targeted against Pofut1 transcripts were assayed for Delta1-induced Notch signaling as in B. D, binding of Delta1-Fc to parental Lec1 (filled squares), HY4 (open circles), HN2 (open triangles), and HN9 (open diamonds) in RNAi-targeted cells analyzed by flow cytometry. MFI is given after subtraction of MFI for secondary antibody alone. E, binding of Jagged1-Fc to Lec1 (filled squares), HY4 (open circles), HN2 (open triangles), and HN9 (open diamonds) by flow cytometry analyzed as in D. F, ligand-independent signaling in Lec1 cells with RNAi-targeted Pofut1 and parental Lec1 cells following incubation in 4 mM EDTA or 2.5 mM  $\text{CaCl}_2$  for 5 min followed by culture for 8 h. -Fold induction is given as the ratio of normalized luciferase activity in EDTA-treated compared with  $\text{CaCl}_2$ -treated cells. Bars, S.D. ( $n = 4$ ).

cells treated with either of the calcium chelators in calcium-free medium (Fig. S3), showing that all four Notch receptor NECDs had been released.

Ligand-independent Notch signaling was assayed in Lec1 and Lec13 cells using either EDTA or BAPTA at concentrations that gave ~50% maximal signal (Fig. S2). Following calcium chelation, Notch signaling was virtually identical in Lec1 and Lec13 cells under all growth conditions (Fig. 3, D and E). As predicted for signaling induced independently of Notch ligand binding, the presence of fucose did not enhance EDTA- or BAPTA-induced signaling by Lec13 cells. In all cases ligand-independent signaling was markedly inhibited by the presence of DAPT (Fig. 3, D and E). Therefore, the degree of O-fucosylation of endogenous Notch receptors does not significantly affect the level of Notch receptor cell surface expression based on both the binding of Notch NECD antibodies (Figs.

3, A and B, and S3) and on ligand-independent Notch signaling (Fig. 3, D and E).

To investigate the mechanism by which fucose supplementation rescues Notch signaling in Lec13 cells, ligand binding was examined by flow cytometry. Lec13 cells grown in the presence or absence of 1 mM galactose had significantly reduced binding of Delta1 and Jagged1 ligands (Fig. 3, F and G). By contrast, Lec13 cells grown in fucose bound Jagged1 and Delta1 similarly to Lec1 cells under each growth condition (Fig. 3, F and G). This result was confirmed using antibodies directed against Delta1 or Jagged1 rather than antibodies directed against the Fc of the chimeric proteins (Fig. S4). In both types of experiment, there was significant Notch ligand binding to Lec13 cells in nonsupplemented medium. This did not correlate with the more significantly decreased Notch signaling in Lec13 cells (Fig. 2), suggesting that functional Notch ligand binding which occurs between juxtaposed cell membranes is not completely reflected by the binding of cell-free, soluble ligands. Nevertheless, it is apparent that O-fucose on Notch receptors is necessary for optimal ligand binding as well as Notch signaling by the canonical pathway.

**siRNA Targeted to Pofut1 Reduces Notch Ligand Binding and Ligand-dependent Notch Signaling**—To investigate roles for Pofut1 in Notch signaling in CHO cells, siRNA sequences designed to reduce

Pofut1 levels were transfected into Lec1 CHO cells to produce stable transfectant populations. Northern blot analyses confirmed the reduction or virtual elimination of Pofut1 transcripts and Western blot analysis confirmed the corresponding reduction in Pofut1 protein (Fig. 4A). Cells with low to undetectable Pofut1 levels (HN2 and HN9, which contain two RNAi constructs) and cells with an intermediate level of Pofut1 (HY4, which contains only one siRNA construct) were chosen for further analysis.

Jagged1-induced Notch signaling was reduced 3–6-fold in HN2 and HN9 cells and ~1.5-fold in HY4 (Fig. 4B). Results were similar for Delta1-induced Notch signaling (Fig. 4C), with HY4 cells exhibiting a smaller reduction than HN2 or HN9. Lec1 cells with reduced Pofut1 were also tested for their ability to bind to soluble Notch ligands by flow cytometry. Based on MFI, the cells expressing Pofut1 siRNA bound ~30–40% less

## Notch Receptors Lacking O-Fucose May Signal

Delta1 and 25–40% less Jagged1 than parental Lec1 cells (Fig. 4, *D* and *E*). The reduction in ligand binding was not due to reduced expression of Notch receptors at the cell surface, as determined by the ligand-independent Notch signaling assay (Fig. 4*F*) and by flow cytometry using antibodies to Notch2, Notch3, and Notch4 ECD (not shown). This result suggests that the loss or reduction of the Pofut1 fucosyltransferase does not appreciably affect the expression of Notch receptors at the sur-

face of CHO cells. This point was investigated in depth using Pofut1-null ES cells.

**ES Cells Lacking Pofut1 Stably Express Cell Surface Notch Receptors but Do Not Bind Notch Ligands or Transduce Notch Signals**—ES cells derived from Pofut1-null embryos provide a powerful tool to study Notch signaling in the complete absence of Pofut1. Pofut1<sup>-/-</sup> ES cells were previously shown to express transcripts from the mutant allele (39). However, these transcripts encode a small peptide, and Western analysis proved that the Pofut1 protein is absent from Pofut1<sup>-/-</sup> cells (see Fig. 8*A*). In addition, these cells have no Pofut1 enzyme activity (41) and Table 1).

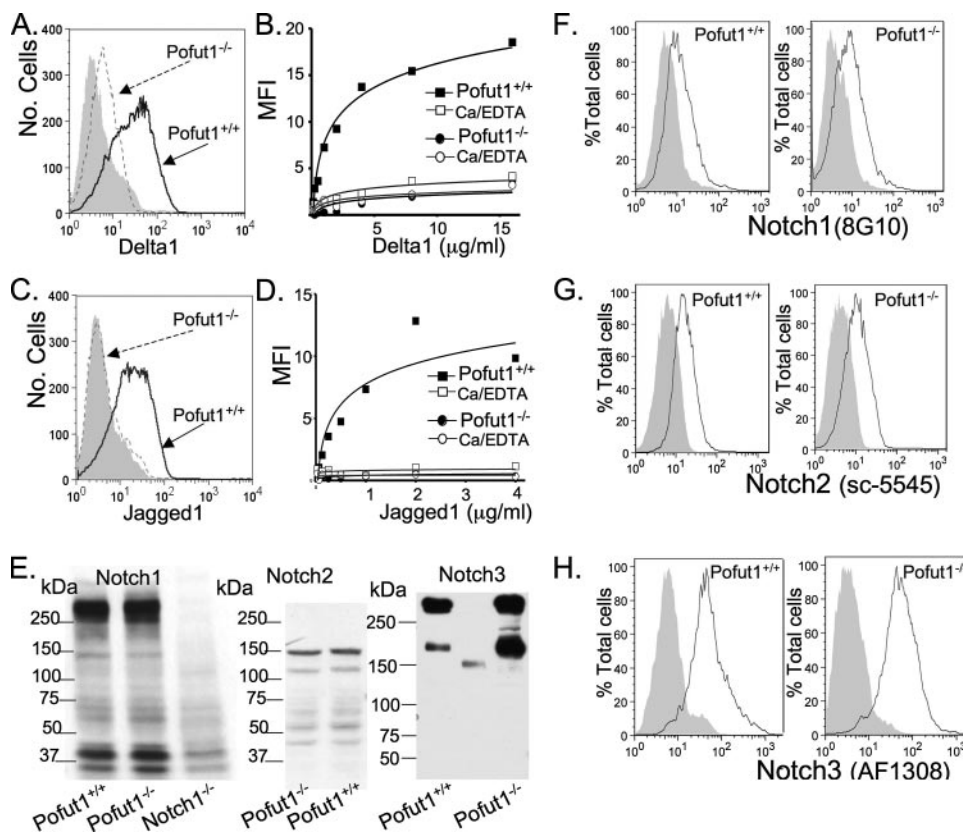
ES cells were examined for their ability to bind Notch ligands by flow cytometry. As observed with CHO cells, Pofut1<sup>+/+</sup> ES cells bound soluble Jagged1 and Delta1 in a calcium-dependent manner inhibited by the addition of EDTA to the binding buffer (Fig. 5, *A* and *C*). Notch ligand binding was concentration-dependent (Fig. 5, *B* and *D*). By contrast, Pofut1<sup>-/-</sup> ES cells did not bind Notch ligands (Fig. 5, *A–D*). The same results were obtained with four independent Pofut1<sup>-/-</sup> ES

**TABLE 1**

### Pofut1 and Gal-T activity of cell extracts

Glycosyltransferase assays of cell lysates were performed as described under "Materials and Methods." Specific activity is in nmol/mg protein/h. Values are the average of 2–3 assays.

	Pofut1 nmol/mg protein/h	$\beta$ 4Gal-T nmol/mg protein/h	Pofut1/ GalT %
Pofut1 <sup>+/+</sup> + vector	1.5	16.5	9.1
Pofut1 <sup>+/+</sup> + Pofut1	18.3	10.3	178
Pofut1 <sup>+/+</sup> + Pofut1 R245A	0.21	13.8	1.5
Pofut1 <sup>-/-</sup> + vector	0.3	14.3	2
Pofut1 <sup>-/-</sup> + Pofut1	20.5	14.3	143
Pofut1 <sup>-/-</sup> + Pofut1 R245A	<0.1	11.4	<1



**FIGURE 5. ES cells lacking Pofut1 are deficient in Notch ligand binding.** *A*, flow cytometric analysis of soluble Delta1-Fc (4  $\mu$ g/ml) binding to Pofut1<sup>+/+</sup> (solid line) and Pofut1<sup>-/-</sup> (dashed line) ES cells. The shaded profile is secondary antibody alone. *B*, Delta1-Fc binding to Pofut1<sup>+/+</sup> (squares) and Pofut1<sup>-/-</sup> (circles) ES cells in buffer containing 1 mM CaCl<sub>2</sub> (solid) or 1 mM CaCl<sub>2</sub> and 5 mM EDTA (open) analyzed by flow cytometry. Data are plotted as MFI minus MFI of secondary antibody alone. *C*, flow cytometric analysis of soluble Jagged1-Fc (2  $\mu$ g/ml) binding to Pofut1<sup>+/+</sup> (solid line) and Pofut1<sup>-/-</sup> (dashed line) ES cells as in *A*. *D*, soluble Jagged1 binding to Pofut1<sup>+/+</sup> (squares) and Pofut1<sup>-/-</sup> (circles) ES cells in buffer containing 1 mM CaCl<sub>2</sub> (solid) or 1 mM CaCl<sub>2</sub> and 5 mM EDTA (open) determined by flow cytometry as in *B*. *E*, Notch1, Notch2, and Notch3 in Pofut1<sup>-/-</sup> ES cells. Western analysis of lysates from Pofut1<sup>+/+</sup>, Pofut1<sup>-/-</sup>, and Notch1<sup>-/-</sup> 290-2 ES cells (100  $\mu$ g of protein) was performed using anti-Notch1 ECD antibody 8G10 (1:500). Pofut1<sup>+/+</sup> and Pofut1<sup>-/-</sup> cell lysates (50  $\mu$ g of protein) were probed with anti-Notch2 Ab sc-5545 (1:500). Pofut1<sup>+/+</sup> and Pofut1<sup>-/-</sup> cell lysates (44  $\mu$ g of protein) were probed with anti-Notch3 Ab 5E1 (1:500 culture medium). Molecular mass markers are in kDa. *F–H*, Pofut1<sup>+/+</sup> and Pofut1<sup>-/-</sup> ES cells were incubated with antibodies to the ECD of Notch1 (clone 8G10), Notch2 (sc-5545), or Notch3 (AF1308) and analyzed by flow cytometry. Secondary antibody alone is shaded in each profile.

lines compared with independent control Pofut1<sup>+/+</sup> and Pofut1<sup>-/-</sup> lines (Fig. S5).

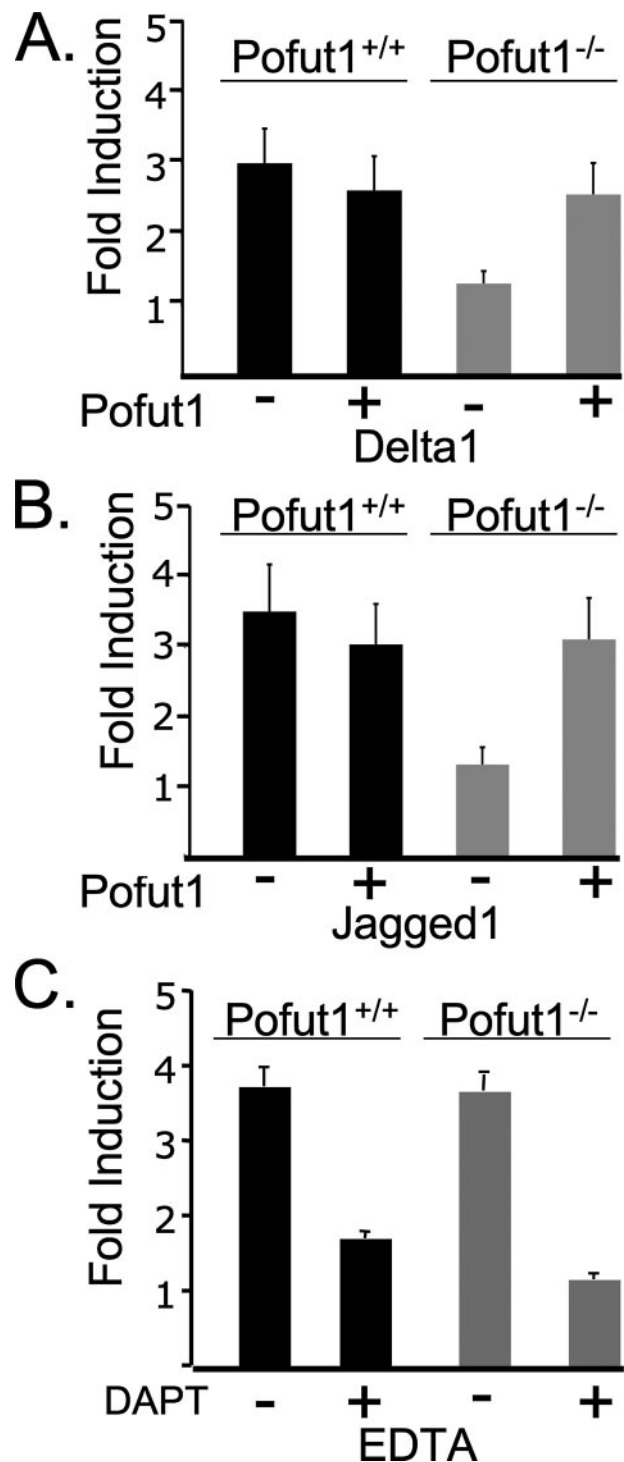
To determine whether the lack of ligand binding to Pofut1<sup>-/-</sup> cells might reflect a reduced number of Notch receptors at the cell surface, flow cytometry with antibodies to NECD was performed. Commercial antibodies to all four Notch receptors bound to wild type ES cells, and independent specificity data were available for Notch1, Notch2, and Notch3 anti-NECD antibodies (Fig. 1*I*) (52–54). Western analysis with anti-Notch1 ECD antibody 8G10 showed equivalent expression of full-length Notch1 in Pofut1<sup>+/+</sup> and Pofut1<sup>-/-</sup> cells and confirmed that Notch1-null ES cells do not express Notch1 (Fig. 5*E*). Monoclonal Ab 5E1 detected equivalent amounts of Notch3 in Pofut1<sup>+/+</sup> and Pofut1<sup>-/-</sup> whole cell lysates (Fig. 5*E*) and was highly specific for full-length Notch3 (~300 kDa) and Notch3 extracellular domain (~180 kDa), as observed for human Notch3 (46). Notch2 antibody sc-5545 detected the extracellular domain as a major species as observed by others (55), which was equivalent in both Pofut1<sup>+/+</sup> and Pofut1<sup>-/-</sup> lysates (Fig. 5*E*). Flow cytometry using anti-NECD antibodies revealed very similar levels of Notch1, Notch2, and Notch3 receptors expressed on the surface of wild type *versus* Pofut1-

null ES cells (Fig. 5, *F–H*). Similar results were found with the anti-Notch3 monoclonal antibody 5E1 (see Fig. 7A). The slightly broader binding curves obtained with ES cells lacking Pofut1 were shown by scatter analysis to be due to variation in cell size. Significantly, this result also indicates that the lack of Pofut1 does not alter recognition by anti-NECD antibodies to three different Notch receptors.

We previously showed that *Pofut1*<sup>-/-</sup> ES cells do not respond to Delta1 or Jagged1 ligands and are rescued by transient expression of a Pofut1 cDNA (39). Additional data confirm these observations and the fact that overexpression of the Pofut1 cDNA did not enhance or inhibit Notch signaling in *Pofut1*<sup>+/+</sup> ES cells (Fig. 6, *A* and *B*). The reduced Notch signaling of *Pofut1*<sup>-/-</sup> ES cells was not due to decreased Notch receptor cell surface expression in the absence of Pofut1, because ligand-independent Notch signaling was equivalent in *Pofut1*<sup>+/+</sup> and *Pofut1*<sup>-/-</sup> ES cells (Fig. 6C), consistent with the results obtained by flow cytometry using anti-Notch NECD antibodies (Fig. 5, *F–H*). Taken together, the results obtained with *Pofut1*<sup>-/-</sup> ES cells and Pofut1-targeted CHO cells show that Pofut1 is not required for stable cell surface Notch receptor expression in cultured mammalian cells, but it is necessary for optimal ligand binding and Notch signaling.

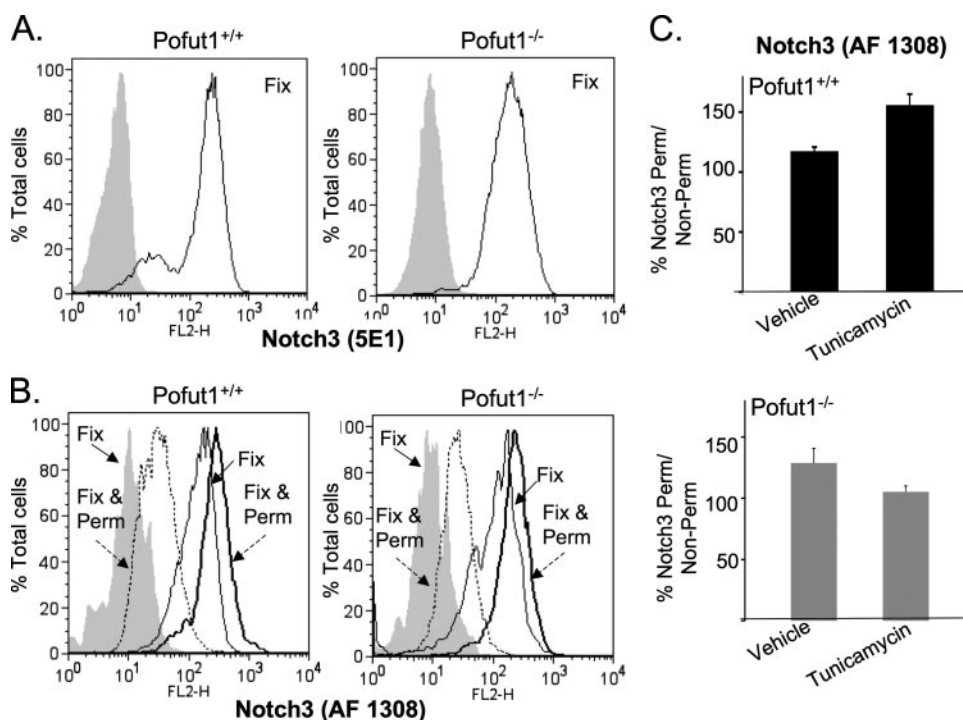
**Intracellular Notch in Pofut1 Wild Type and Null ES Cells**—Ofut1 removal from *Drosophila* S2 cells leads to intracellular Notch accumulation (16–18, 37). Murine ES cells lacking Pofut1 were used to determine whether this also occurs in mammalian cells. Notch3 levels were compared in *Pofut1*<sup>+/+</sup> and *Pofut1*<sup>-/-</sup> cells by flow cytometry of permeabilized *versus* nonpermeabilized cells using anti-Notch3 ECD antibodies from two sources. Cell surface Notch3 was determined after fixation using anti-Notch3 antibodies 5E1 and AF1308 (Fig. 7, *A* and *B*). Both antibodies bound well to fixed cells, as expected from flow cytometry of cells treated with sodium azide. Total Notch3 was determined after fixation and permeabilization using anti-Notch3 AF1308. A small increase in anti-Notch3 antibody binding was observed in both *Pofut1*<sup>+/+</sup> and *Pofut1*<sup>-/-</sup> cells after permeabilization (Fig. 7B). This increase was slightly higher in *Pofut1*<sup>-/-</sup> cells, consistent with a small amount of intracellular accumulation of Notch3 in *Pofut1*<sup>-/-</sup> cells. Anti-Notch3 antibody 5E1 bound less after permeabilization (not shown), suggesting that its epitope was partly inactivated by the permeabilization conditions. Nevertheless, *Pofut1*<sup>+/+</sup> and *Pofut1*<sup>-/-</sup> cells exhibited similar profiles after permeabilization.

In a second experiment, cells were compared for cell surface and total Notch3 levels after treatment with tunicamycin, which increases misfolding of glycoproteins in the endoplasmic reticulum and their retention or degradation (56). ES cells grown in the presence or absence of tunicamycin were incubated with anti-Notch3 AF1308 or control secondary antibodies at room temperature in binding buffer with azide, washed after 30 min, and incubated with secondary antibody. After washing, the cells were divided into two aliquots. Both aliquots were fixed, and one aliquot was fixed and permeabilized as above, and then both were incubated with anti-Notch3 AF1308 or PE-labeled secondary antibody, washed, and incubated with PE-labeled secondary antibodies. Flow cytometry was per-



**FIGURE 6. *Pofut1*<sup>-/-</sup> and *Pofut1*<sup>+/+</sup> ES cells exhibit equivalent ligand-independent Notch signaling.** *A*, Delta1-induced Notch signaling is reduced in *Pofut1*<sup>-/-</sup> ES cells and rescued by Pofut1 cDNA. Bars, S.D. (*n* = 10, including 4 data points from Shi *et al.* (39)). *B*, Jagged1-dependent signaling is reduced in *Pofut1*<sup>-/-</sup> ES cells and rescued by Pofut1 cDNA. Bars, S.D. (*n* = 10, including 4 data points from Shi *et al.* (39)). *C*, ligand-independent Notch signaling in *Pofut1*<sup>-/-</sup> and *Pofut1*<sup>+/+</sup> ES cells induced by incubation in 4 mM EDTA for 5 min compared with signaling in the presence of 2.5 mM CaCl<sub>2</sub> with or without the  $\gamma$ -secretase inhibitor DAPT (500 nM). -Fold induction is the ratio of normalized EDTA-treated to CaCl<sub>2</sub>-treated cells. Error bars, S.D. (*n* = 4).

formed to determine cell surface Notch3 *versus* cell surface plus internal Notch3. Based on MFI, permeabilized *Pofut1*<sup>+/+</sup> ES cells bound ~17% more Notch3 than nonpermeabilized cells.



**FIGURE 7. Intracellular Notch in *Pofut1*<sup>-/-</sup> ES cells.** A, *Pofut1*<sup>+/+</sup> and *Pofut1*<sup>-/-</sup> ES cells were fixed and analyzed by flow cytometry using anti-Notch3 antibody 5E1 (solid line) or secondary antibody alone (shaded profile). B, *Pofut1*<sup>+/+</sup> and *Pofut1*<sup>-/-</sup> ES cells were fixed or fixed and permeabilized and analyzed by flow cytometry using anti-Notch3 antibody AF1308 or secondary antibody as indicated. C, *Pofut1*<sup>+/+</sup> and *Pofut1*<sup>-/-</sup> ES cells treated with DMSO (vehicle) or 2 μg/ml tunicamycin were incubated with anti-Notch3 ECD antibody AF1308 or secondary antibody, fixed, or fixed and permeabilized and subjected to flow cytometry. The percentage of background-subtracted MFI for permeabilized compared with nonpermeabilized cells is plotted. Bars, the range of values in two experiments.

When *Pofut1*<sup>+/+</sup> ES cells were treated with tunicamycin, Notch3 levels in permeabilized cells increased (Fig. 7C), reflecting an increase in the intracellular concentration of Notch3. Nonpermeabilized *Pofut1*<sup>-/-</sup> cells had similar levels of cell surface-bound anti-Notch3 compared with wild type ES cells, consistent with data in Fig. 7, A and B, and data from unfixed cells (Fig. 5). The MFI of anti-Notch3 binding to *Pofut1*<sup>-/-</sup> cells increased by ~30% following permeabilization. Thus, somewhat more intracellular Notch3 was found constitutively in ES cells lacking Pofut1 as seen in Fig. 7B. Interestingly, however, tunicamycin treatment did not increase the MFI of permeabilized *Pofut1*<sup>-/-</sup> cells (Fig. 7C). Therefore, although ES cells lacking Pofut1 have somewhat more intracellular Notch3 than wild type ES cells, this is not accompanied by a reduction in stable expression of Notch3 (or other Notch receptors shown previously) at the surface of *Pofut1*<sup>-/-</sup> cells.

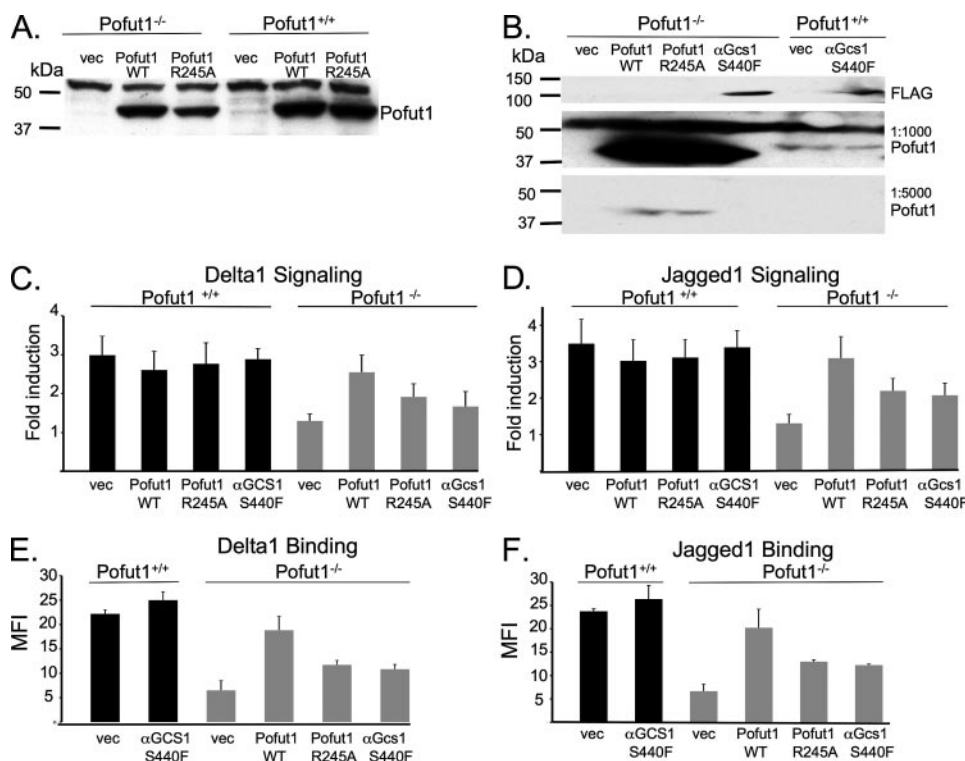
**Overexpression of Inactive Pofut1 or an Unrelated Inactive ER Enzyme Partially Rescues Notch Signaling in *Pofut1*<sup>-/-</sup> ES Cells**—In *Drosophila*, Ofut1 exhibits a chaperone activity for Notch that is retained by a fucosylation-defective Pofut1/Ofut1 mutant R245A (16, 18). To investigate the ability of the mouse Pofut1 R245A fucosyltransferase-defective mutant to support Notch signaling in mammalian cells, the relevant cDNAs were transfected into *Pofut1*<sup>+/+</sup> or *Pofut1*<sup>-/-</sup> ES cells, and the cells were assayed for Notch signaling and ligand binding. Western blot analysis (Fig. 8A) and a fucosyltransferase assay (Table 1) confirmed that both constructs were overexpressed in ES cells and that Pofut1 R245A had no *in vitro* fucosyltransferase activity. Endogenous Pofut1 is present in low amounts in wild type ES cells compared with the enormous increase in Pofut1 levels following transfection of a Pofut1 cDNA (Fig. 8A). It is notable that the increase in Pofut1 activity is not as great (Table 1) as the increase in protein (Fig. 8A). It also appears that Pofut1 R245A has a dominant negative effect on wild type Pofut1, since Pofut1 activity in *Pofut1*<sup>+/+</sup> cells expressing Pofut1 R245A was markedly reduced in the *in vitro* assay (Table 1). Nevertheless, Pofut1 activity in the ES cells was sufficient to support Notch signaling (see below).

Overexpression of wild type Pofut1 rescued Notch signaling in *Pofut1*<sup>-/-</sup> cells (Fig. 8, C and D), as reported previously (39). In addition, overexpression of the mutant Pofut1 R245A also led to increased ligand-induced Notch signaling (Fig. 8, C and D), although this was ~40% less than that obtained with wild type Pofut1. Neither wild type Pofut1 nor Pofut1 R245A overexpression significantly altered ligand-induced signaling in *Pofut1*<sup>+/+</sup> ES cells (Table 2). This is of interest, because in *Drosophila*, overexpression of wild type Ofut1 inhibits Notch signaling (12, 17, 18). The specificity of the facilitation of Notch signaling by Pofut1 R245A was investigated by overexpressing an inactive mutant of an unrelated, ER-localized α-glucosidase 1 termed α-Gcs1 S440F (45) (Fig. 8B). The overexpression of α-Gcs1 S440F enhanced ligand-induced Notch signaling to a similar extent as Pofut1 R245A (Fig. 8, C and D, and Table 2). Therefore, the enhanced Notch signaling observed following overexpression of Pofut1 R245A was not specific to inactive Pofut1.

Overexpression of wild type Pofut1 corrected ligand binding to *Pofut1*<sup>-/-</sup> ES cells as expected (Fig. 8, E and F). The Pofut1 R245A fucosyltransferase mutant also improved Notch ligand binding but significantly less than wild type Pofut1 (Fig. 8, E and F). Once again, overexpression of the unrelated cDNA α-Gcs1 S440F caused essentially the same partial correction in ligand binding as observed with Pofut1 R245A. However, Notch ligand binding to wild type ES cells was not significantly altered by overexpression of wild type Pofut1 or the Pofut1 R245A mutant (Fig. 8, E and F). Similarly, the cell surface levels of Notch1 or Notch3 receptors were not altered by overexpression of wild type Pofut1 or Pofut1 R245A in ES cells (not shown).

**DISCUSSION**

Pofut1 is an essential component of the canonical Notch signaling pathway in mammals during postgastrulation develop-



**FIGURE 8. Overexpression of inactive ER enzymes partially rescues Notch ligand binding and signaling in *Pofut1*<sup>-/-</sup> ES cells.** A, Western blot analysis of whole cell lysates of *Pofut1*<sup>-/-</sup> ES cells transiently expressing vector, a *Pofut1* cDNA (*Pofut1* WT), or a fucosyltransferase mutant cDNA (*Pofut1* R245A) using anti-*Pofut1* antibody. The blot was exposed for a sufficient time to show *Pofut1* in *Pofut1*<sup>+/+</sup> ES cells. B, Western analysis after transient transfection of the inactive mutant  $\alpha$ -glucosidase S440F ( $\alpha$ -Gcs1 S440F) detected by anti-FLAG antibody (top). The lower panels show the same blot reprobed using different dilutions of *Pofut1* antibody for detection of endogenous *Pofut1* (1:1000) and transfected *Pofut1* wild type or R245A (1:5000). C and D, *Pofut1*<sup>+/+</sup> and *Pofut1*<sup>-/-</sup> ES cells were transfected with vector, *Pofut1* wild type, *Pofut1* R245A, or  $\alpha$ -Gcs1 S440F, TP1 Notch reporter, and pRL-TK-*Renilla* luciferase. Following co-culture with Delta1/L, Jagged1/L, or control L cells, luciferase activities were assayed, and -fold induction was calculated. Bars, S.D. ( $n = 4$  for  $\alpha$ -Gcs1 S440F,  $n = 10$  for others). Statistical comparisons are given in Table 2. E and F, *Pofut1*<sup>-/-</sup> and wild type ES cells were transfected as in C and D and examined for binding of Delta1-Fc (4  $\mu$ g/ml) or Jagged1-Fc (1  $\mu$ g/ml) by flow cytometry. The MFI minus MFI for secondary antibody alone was averaged. Bars, the range of MFI values in two experiments.

**TABLE 2**  
**Notch Signaling in *Pofut1*<sup>-/-</sup> ES cells**

The ratio of the average Notch signaling level in ES cell transfectants in the left column is compared with those in the right column from the data in Fig. 8, C and D.  $p$  values in parentheses are based on the two-tailed Student's  $t$  test. Vec, vector; +/+, *Pofut1*<sup>+/+</sup> ES cells; -/-, *Pofut1*<sup>-/-</sup> ES cells; cDNA transfected is given in parentheses.

ES cell transfectants	Average Notch signaling ratio	
	Delta1 ( $p$ )	Jagged1 ( $p$ )
+/+ (+Vec) : -/- (+Vec)	2.30 (<0.001)	2.70 (<0.001)
+/+ (+Vec) : -/- (+Pofut1)	1.17 (0.05)	1.12 (0.18)
+/+ (+Vec) : -/- (+Pofut1 R245A)	1.56 (<0.001)	1.59 (<0.001)
+/+ (+Vec) : -/- (+ $\alpha$ -Gcs1 S440F)	1.79 (<0.001)	1.68 (0.002)
-/- (+Pofut1) : -/- (+Pofut1 R245A)	1.33 (0.002)	1.41 (0.001)
-/- (+Pofut1) : -/- (+ $\alpha$ -Gcs1 S440F)	1.53 (0.004)	1.49 (0.008)
-/- (+Pofut1 R245A) : -/- (+ $\alpha$ -Gcs1 S440F)	1.06 (0.55)	1.15 (0.25)

ment (14, 39, 57). Here we have investigated the molecular basis for this requirement in experiments that separately examined roles for O-fucose modification of Notch receptors *versus* a role for *Pofut1* as a chaperone for Notch receptors. We show that Lec13 CHO cells that have little GDP-fucose (32, 33, 35) have normal *Pofut1* levels but nevertheless exhibit poor Delta1- and Jagged1-induced Notch signaling. The addition of fucose, but

not galactose, to the medium restores Notch signaling to control levels for both ligands. Notch ligand binding was also optimal only when fucose was added to the medium. Since Lec13 cells express the same amount of active *Pofut1* under all conditions tested, it is fucose that rescues ligand binding and Notch signaling in Lec13 cells. Moreover, this is not because fucose increases endogenous Notch receptor levels at the CHO cell surface. Therefore, fucose on Notch EGF repeats may facilitate appropriate folding or recognition by Notch ligands. However, simple sugars, including 5 mM L-fucose, do not inhibit ligand binding,<sup>9</sup> as also observed with *Drosophila* Notch (29). This suggests that canonical ligands must recognize aspects of Notch EGF structure or conformation, perhaps with a contribution from O-fucose. Interestingly, fucose marginally enhanced cell surface expression following overexpression of Notch1 in Lec13 cells as well as improving the secretion of overexpressed Notch1 EGF-(1–18) from Lec13 cells,<sup>10</sup> similar to effects observed with overexpressed thrombospondin repeats and O-fucosylation by *Pofut2* (58, 59). Therefore, under the stress of overexpression, O-fucosylation appears to assist trafficking of Notch receptors

through the secretory pathway in mammalian cells, but this was not observed for endogenous Notch in CHO or ES cells. In *Drosophila* mutant embryos lacking *Gmd* and thus GDP-fucose, as in Lec13 CHO cells, Delta-induced Notch signaling functions well during early neurogenesis (18). Whether Notch function is optimal in *gmd*<sup>-</sup> cells cannot be tested in *Drosophila*, which lacks the enzymes that convert fucose to GDP-fucose (60).

When *Pofut1* levels were severely reduced by siRNA targeting in CHO cells or eliminated by targeted mutation in *Pofut1*<sup>-/-</sup> ES cells, Notch signaling and canonical Notch ligand binding were lost. Importantly, however, and in stark contrast to the situation in both *Drosophila* S2 cells (16) and cells of the wing disc (16–18, 37), the lack of *Pofut1* did not lead to a significant decrease in the cell surface expression of mammalian Notch receptors. Although there was a small amount of intracellular accumulation of Notch3 in ES cells lacking *Pofut1*, this was not accompanied by a significant decrease in cell surface expression. Therefore, *Pofut1* is not an essential chaperone for

<sup>9</sup> C. Ge and P. Stanley, unpublished observations.

<sup>10</sup> Y. Tashima and P. Stanley, unpublished observations.

mammalian Notch receptors to traffic to, or to be stably expressed at, the surface of CHO or murine ES cells. Experiments in cultured *Drosophila* wing discs also suggest that Ofut1 may not be required for trafficking of Notch to the cell surface, although it is required for stable cell surface expression of Notch (17, 37). However, there are technical concerns that may affect the interpretation of these experiments (18, 61, 62). Interestingly, *Drosophila* Notch lacking O-glucose in the *rumi* mutant is functionally inactive at 25 °C but is expressed well at the cell surface (63).

Similar to *Drosophila* Notch, mammalian Notch receptors synthesized in the absence of Pofut1 do not bind to Notch ligands (16, 29). This suggests defective folding although recognition by anti-ECD antibodies to three Notch receptors on the surface of ES and CHO cells was not impaired. It may also suggest participation of, although not an absolute requirement for, O-fucose in Notch recognition by ligand. Thus, when human Notch1 ligand binding domain fragments are made in *Escherichia coli* and folded well enough to bind calcium (64, 65), ligand binding is of very low affinity compared with binding kinetics obtained for mammalian Notch receptors that are post-translationally modified (Fig. 1) (25). In addition, only partial rescue of ligand binding to Notch receptors was achieved in *Pofut1*<sup>-/-</sup> ES cells by the inactive Pofut1 R245A mutant. Importantly, this rescue was not specific for Pofut1, since ER-localized, inactive,  $\alpha$ -glucosidase I, a protein completely unrelated to Pofut1, achieved a similar degree of rescue for both ligand binding and Notch signaling. Therefore, the rescue ability of Pofut1 R245A was not due to residual enzyme activity or to specific chaperoning of mammalian Notch receptors. Rather, nonspecific effects were responsible, most likely due to the up-regulation of chaperone activities in the ER or ER-cis-Golgi (ERGIC) that occurs when cells overexpress an ER glycoprotein (66).

The situation in *Drosophila* is different. Expression of a *Drosophila* Ofut1 R245A mutant from the endogenous *OFUT1* locus rescues Notch signaling during early neurogenesis (18). In the wing disc, the phenotype generated by this mutation is similar to that induced by loss of Fringe activity. The authors (18) conclude that fucose on *Drosophila* Notch serves primarily as an acceptor for Fringe and that Ofut1 is required as a specific chaperone for the proper folding of Notch. It is curious that Ofut1 is apparently required neither for the folding/functions of Notch ligands (12, 17), which also acquire O-fucose (67), nor of Crumbs, which contains 30 EGF repeats, many of which have the consensus for O-fucosylation (16). It will be important to further investigate these questions *in vivo* in mammals. In Lec13 CHO cells, Jagged1-induced Notch signaling is increased by fucose rescue rather than decreased, as would be predicted if O-fucose on Notch functions only as a scaffold for Fringe (15).

In summary, in contrast to *Drosophila* Ofut1, mammalian Pofut1 is not an essential chaperone for stable, cell surface expression of Notch receptors. However, cell surface Notch receptors synthesized in the absence of Pofut1 do not bind ligands, presumably because they are improperly folded. Overexpression of an unrelated ER glycoprotein allows the synthesis of functional Notch in *Pofut1*<sup>-/-</sup> ES cells. However, the addi-

tion of O-fucose is required for *optimal* ligand binding and ligand-induced Notch signaling. In mammals, Notch made in the absence of Pofut1 may never occur *in vivo*, since Pofut1 appears to be ubiquitously expressed in mammalian cells and tissues (14, 68). By contrast, Ofut1 in *Drosophila* is differentially expressed after embryonic development (12, 13). Another difference between *Drosophila* and mammalian Notch receptors is that ectopic expression of wild type Ofut1 inhibits Notch signaling (16–18), whereas overexpression of active or inactive Pofut1 in wild type ES cells does not inhibit or promote Notch signaling (Fig. 8) (39). Additionally, it has been suggested that most *Drosophila* Notch is not cleaved by furin in the Golgi, whereas mammalian Notch receptors are expressed primarily as heterodimers at the cell surface, leading to the proposal that the mechanism of Notch activation by ligands may differ in *Drosophila* and mammals (31). Finally, the complement of ER chaperones and the temperature of growth are quite different in *Drosophila* and mammals. Some combination of these effects presumably allows stable cell surface expression of mammalian Notch receptors in the absence of Pofut1. The combined results emphasize the fact that conclusions drawn from studies of *Drosophila* Notch (18, 61, 62), cannot necessarily be applied to mammalian Notch receptors. Another example of a substantial difference between mammals and *Drosophila* is in the *in vivo* consequences of eliminating the O-fucose site in the Notch1 ligand binding domain. In *Drosophila*, this mutation leads to a more active Notch (27), whereas in mammals, it leads to a weak (hypomorphic) Notch1 (69).

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